IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: REIMANN=1

In re Application of:	)	Examiner: A. Beckerleg
Jorg REIMANN et al	)	Art Unit: 1632
Appln. No.: 09/241,595	)	Washington, D.C.
Confirmation No.: 8928	)	
Filed: February 2, 1999	)	
For: DELIVERY OF IMMUNOGENIC MOLECULES VIA HBsAg PARTICLES	)	

DECLARATION UNDER 37 CFR '1.132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Jorg REIMANN, hereby declare and state as follows:

I am an inventor of the above-identified application, and my educational and professional experience is presented in the curriculum vitae attached hereto.

I understand that there is an outstanding lack of enablement rejection under 35 U.S.C. §112, first paragraph. The experimental results described below, which address some of the enablement issues raised by the examiner, were either conducted by me or under my direct supervision, and I can attest of my own personal knowledge that all the results reported herein are true and accurate.

I. The issue that the skilled artisan would not have predicted success in making HbsAg particles which encapsulate a hydrophobic or insoluble protein or peptide using the described methodology of incubating the two ingredients in aqueous solution (Office Action of June 5, 2002, paragraph bridging pages 3 and 4).

Exhibit A attached hereto shows the results of stimulating the immune response (specifically the CTL response) in BALB/c or C57Bl/6 mice with HBsAg particles loaded with a hydrophobic HBc/eAg peptide having the sequence ***LVV***SY***V***NTNMG***L***K***F***RQ***LLWF*** (bold and italic letters represent hydrophobic amino acids, bestowing the peptide with its hydrophobicity). The HBsAg particles were loaded with the peptide using the same methodology described in the specification, i.e., by simple mixing and incubation of the two ingredients. It is clear from the results shown in Exhibit A that, when inoculating the mice with empty HBsAg particles or with the peptide alone, a very weak or no CTL immune response was induced. However, HBsAg particles loaded with either 5μg or 50μg of the hydrophobic peptide resulted in a significant, dose dependent CTL response to the antigen.

Exhibit B attached hereto provides results of a similar assay, except that the HBsAg particles were loaded (by mere mixing and incubation) with an immunostimulatory oligodeoxynucleotide (ODN1826). As shown in Exhibit B, HBsAg particles alone or ODN alone have a very weak (if any) immune CTL stimulating effect, whereas the HBsAg particles loaded with the ODN induced a significant CTL response.

II. The issue of unpredictability in generating an immune response because the working example with IL-2 in the specification shows that HBsAg particles encapsulating IL-2 were ineffective in generating a HBsAg CTL response (Office Action of June 5, 2002, second paragraph on page 5).

Exhibit C attached hereto presents the materials and methods for preparation of HBsAg particles loaded with IL-2 and IFNα and for determination of entrapment. Table 1 below summarizes the entrapment and bioactivity (% from theoretical bioactivity) of IL-2 with different entrapment methods while Table 2 below summarizes the entrapment and bioactivity (% from theoretical bioactivity) of IFNα with different entrapment methods.

Table 1-Entrapment and Bioactivity of IL-2

Prep.	PREPARATION DESCRIPTION	% IL-2 entrapment		% active IL-2
		BY SDS-PAGE	O.D. 280nm	CTLL proliferation bioassay
A.	HBsAg+IL-2: incubation 10 min at 4°C (Wash Retentate fraction)	28.9	36.12	33.3
B.	HBsAg+IL-2: incubation 60 min at R.T (Wash Retentate fraction)	50	24.5	52.1
C.	HBsAg+ IL-2: incubation 10 min at 4°C + 10min Sonication (Wash Retentate fraction)	37.2	35.52	26.7
D.	HBsAg mixed with IL-2 and lyophilized (Wash Retentate fraction)	30.1	47.4	13.3
E.	HBsAg lyophilized, suspended with IL-2 (Wash Retentate fraction)	39.1	30.7	23.1
F.	HBsAg+ IL-2: electroporation 50V 1000 μs (Wash Retentate fraction)	37.3	31.02	16.9
G.	HBsAg+ IL-2: electroporation 500V 1000 μs (Wash Retentate fraction)	24.5	21.06	7.5

Prep.	PREPARATION DESCRIPTION	% IL-2 entrapment		% active IL-2
		BY SDS-PAGE	O.D. 280nm	CTLL proliferation bioassay
H.	HBsAg+ IL-2: 8 cycles of freezing and thawing at 45°C (Filtrate fraction)	26.8	22.12	54.3
I.1	HBsAg+ IL-2: incubation in present of 5% ethanol, at 4°C, for 10 min	26		
I.2	HBsAg+ IL-2: incubation in present of 5% ethanol, at RT, for 10min	35.3		
Control	Control: IL-2 after ultrafiltration (Wash Retentate fraction)	0	4.07	0

The above results in Table 1 show that IL-2 was effectively encapsulated into HBsAg particles without impairing its biological activity. Encapsulation procedures B and H were the most effective in obtaining biologically active IL-2-loaded HBsAg particles. These results provide support for a previously presented argument that the specification's working example regarding IL-2 failed to show CTL response only because of unsuccessful loading conditions which led to inactivation of the cytokine. However, by applying the same methodology (i.e., mere mixing and incubation of the two ingredients) at a less aggressive temperature (e.g., R.T. in procedure B instead of 45°C) the IL-2 loaded into the particle retained its bioactivity.

Since IL-2 has already been associated in the literature with CTL response (as also admitted by the examiner

on page 5, second paragraph, of the Office Action of June 5, 2002), it is highly expected that the biologically active IL-2 loaded HBsAg particles as demonstrated above are also immunostimulating *in vivo*.

Table 2: entrapment and bioactivity of IFN-α

		% IFN-α entrapment		**% of bioactivity**
Prep.	**Preparation procedure**	**SDS-PAGE (million IU/ml)**	**O.D. 280 nm**	**Zwei Test (million IU/ml)**
A.	HBsAg+IFN-α: , incubation 10 min at 4°C	10.9	8.41	
B.	HBsAg+IFN-α: incubation 60 min at R.T (Wash Retentate fraction)	17.1	16.88	13.3
C.	HBsAg+ IFN-α: incubation 10 min at 4°C + 10 min Sonication	9.7	8.4	
D.	HBsAg mixed with IFN-α and lyophilized		13.6	
E.	HBsAg lyophilized, suspended with IFN-α (Wash Retentate fraction)	12.7	21.53	10.0
F.	HBsAg+ IFN-α: incubation for 60 min at 37°C	12.6	33.86	
G.	HBsAg+ IFN-α: 8 cycles of freezing and thawing at 45°C (Wash Retentate fraction)	14.5	30.58	10.0
Control	INF-α 200 mg/mL			62.5

The results in Table 2 show that IFN-α loaded into HBsAg particles by the above procedures has retained its biological activity (procedure B and E examined) presented as percent from the expected, theoretical bioactivity. Thus, it

is expected that the IFN-α-loaded particles would be effective in stimulating or modulating an immune response.

Furthermore, the results presented in Exhibit D attached hereto show that co-loading of antigenic HBc/eAg-derived peptide with IL-2 facilitates CTL priming to the HBcAg (but not the HBsAg epitope). This means that IL-2 can facilitate priming of a CTL response.

III. The issue that, for a protein which as a result of lower melting temperatures require incubation at temperatures lower than 56°C, one of skill in the art would not be able to predict whether the amount of protein incorporated would be sufficient to modulate any type of immune response.

Even though the specification at page 4 discloses that the temperature of incubation is <u>preferably</u> between about 35°C and about 60°C, and <u>more preferably</u> between about 55°C and about 60°C, this cannot be construed as limiting the temperature of incubation (loading) to only the ranges specified. As shown in Exhibit C and Tables 1 and 2, the same methodology, albeit at lower temperatures than the preferred ranges disclosed in the specification, was also effective in loading the cytokines into the HBsAg particles. Mere change of the temperature cannot be regarded as requiring undue experimentation in practicing the instant invention. In this connection, the examiner also states that the temperatures at which the particles are incubated significantly affects the amount of peptide incorporated. This is irrelevant to the invention as high levels of encapsulation of cytokines into

HBsAg particles are not claimed, but rather the response achieved by the loaded particles, irrespective of whether a higher amount of an immunostimulatory agent could have been loaded into the particles under other conditions. For proteins which as a result of lower melting temperatures require incubation at temperatures less than those described in the specification, the skilled artisan would only need routine experimentation in order to determine the appropriate temperature for obtaining biologically active cytokine-loaded particles as well as to optimize the level of cytokine loaded.

IV. The issue that, while the specification teaches that the purpose of stimulating an immune response such as a CTL response is for vaccination against infectious organisms such as viruses or bacteria, the specification does not provide evidence that stimulating a CTL response would result in protection or treatment of an infection.

At the time the invention was made the skilled artisan would recognize that there is a correlation between CTL responses and disease treatment. As evidence thereof, three articles from among numerous articles in the field describing such a correlation, Kent, S. J., et al., "Detection of simian immunodeficiency virus (SIV)-specific $CD8^+$ T cells in macaques protected from SIV challenge by prior SIV subunit vaccination" *J.Virol.* 70:4941 1996; Rehermann, B., C. et al., "The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response" *Nat.Med.* 2:1104, 1996; and White, K. L., et al., "MHC class I-dependent presentation

of exoerythrocytic antigens to CD8+ T lymphocytes is required for protective immunity against *Plasmodium berghei*" *J.Immunol.* 156:3374, 1996, are attached as Exhibits E1, E2 and E3.

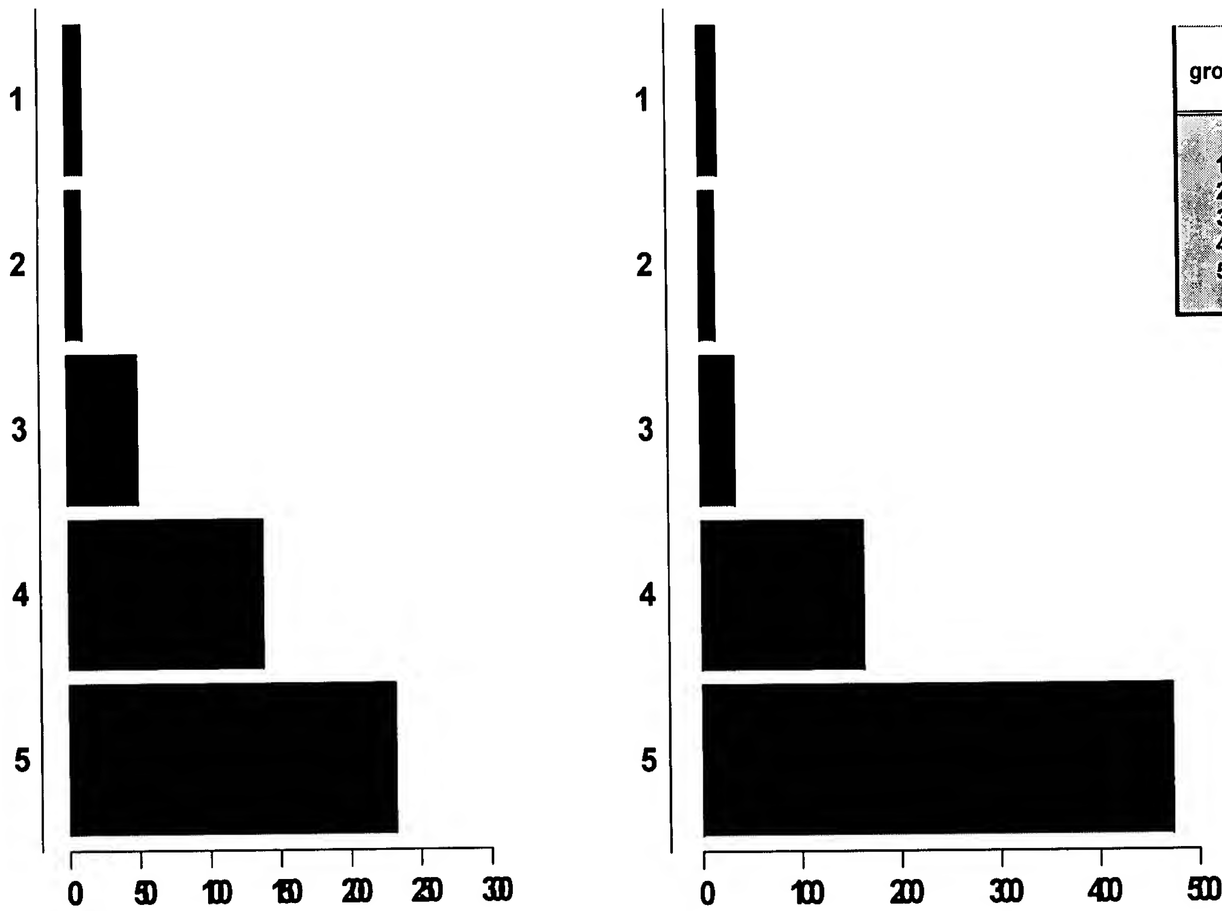
The undersigned declares further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Ulm Nov 1st, 02
Date

Reimann
Jorg REIMANN

BALB/C C57Bl/6

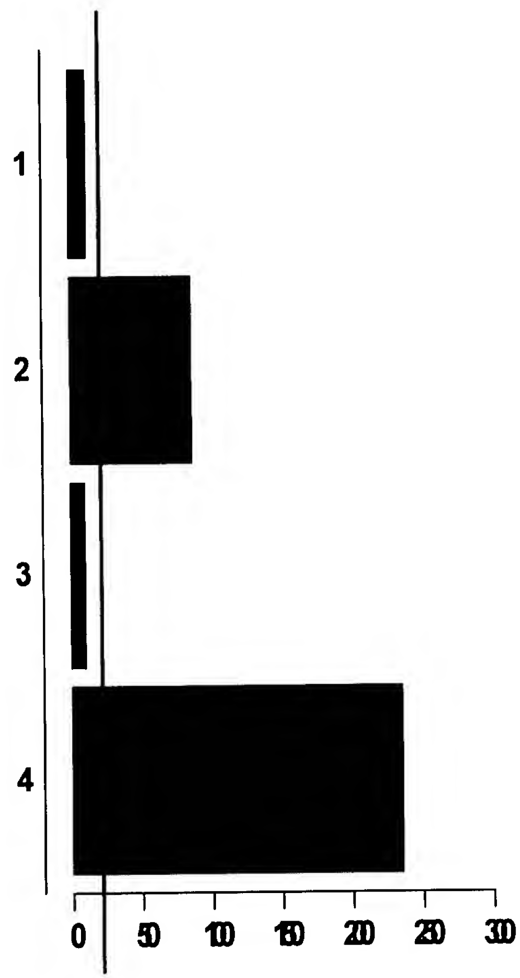
Pep: LVVSYVNTNMGLKFRQLLWF

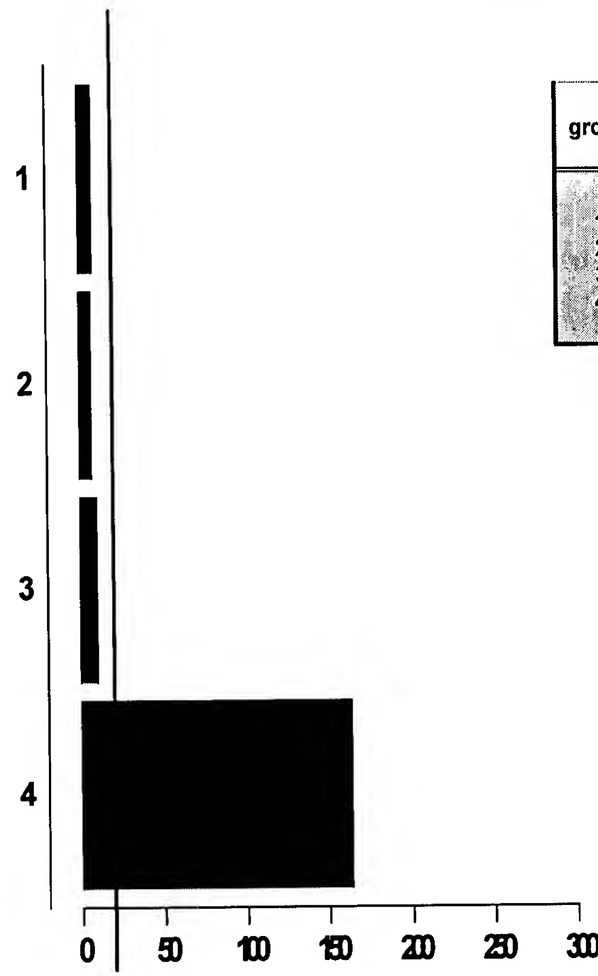


group	Vaccine Antigen I (dose µg)	Vaccine Antigen II (dose µg)	treatment
1	---	---	---
2	HBsAg	---	---
3	Pep (50)	---	---
4	HBsAg	Pep (5)	load
5	HBsAg	Pep (50)	load

HBc/e- specific IFNγ^+ CD8$^+$ T cells /10^5 CD8$^+$ splenic T cells

Exhibit A





ODN1826: TCCATGACGTTCCTGACGTT

group	Vaccine Antigen I (dose µg)	Vaccine Antigen II (dose µg)	treatment
1	---	---	---
2	HBsAg	---	---
3	ODN (10)	---	---
4	HBsAg	ODN (10)	load

HBs- specific IFNγ^+ CD8$^+$ T cells /10^5 CD8$^+$ splenic T cells

Exhibit B

Exhibit C

Preparation of HBsAg particles loaded with IL-2 and INF-α

Materials:

HBsAg derived from *Hansenula* polymorpha (Rhein Biotech, Dusseldorf, Germany; [Diminsky D. *et al.* Vaccine **15**(617):637-647. (1997)] were used as the HBsAg particles (batches maintained in PBS). The particles were mixed with the cytokine (IL-2 or INF-α) at a weight ratio of HBsAg particle:cytokine of 1:1, i.e. for 1mL preparation 200μg of HBsAg particles were mixed with 200μg of cytokine.

Incubation conditions:

For IL-2:

77 μL HBsAg (2.61mg/μL) + 77 μL IL-2 (2.62 mg/μL) were mixed and incubated as described below. PBS (pH 7.2) was added to the mixture to reach a final volume of 1 ml.

For IFNα:

77 μL HBsAg (2.61mg/μL) + 79 μL INF-α (2.53 mg/μL) were mixed and incubated as described below. PBS (pH 7.2) was added to the mixture to reach a final volume of 1 ml.

Encapsulation methods:

A. Incubation of HBsAg particles with the cytokine (IL-2 or IFN-alpha) at 4°C for 10 min.

B. Incubation of HBsAg particles with the cytokine (IL-2 or IFN-alpha) at room temperature (RT) for 60 min.

C. Incubation of HBsAg particles with the cytokine (IL-2 or IFN-alpha) at 4°C for 10 min followed by 10 min of sonication in a bath sonication at 4°C.

D. Co-lyophilization of HBsAg particles and the cytokine (IL-2 or IFN-α). Hydration of the powder with double distilled water (DDW).

E. Lyophilization of HBsAg particles. The entrapment was carried out when the cytokine (IL-2 or IFN-alpha) solution was added to the HBsAg powder.

F. Electroporation of a mixture of HBsAg and cytokine (IL-2 or IFN-α) with the introduction into the solution of the mixed ingredients of two pulses of 50v for 1 msec.

G. Electroporation of a mixture of HBsAg and cytokine (IL-2 or IFN-α) with the introduction into the solution of the mixed ingredients of two pulses of 500v for 1 msec.

H. Eight cycles of freezing at liquid nitrogen and then thawing (at 45°C) of a mixture of HBsAg and the cytokine (IL-2 or INF-α)/

I. Detergents- 5% or 10% of ethyl alcohol was added to a mixture of HBsAg and cytokine (IL-2 or IFN-α) which was then incubated for 10 min. at either 4°C (mixture I(1)) or at RT (mixture I(2)).

J. Incubation of HBsAg particles with IFN-α at 37°C for 60 min.

Unloaded cytokines were removed from the mixture by ultrafiltration using Microsep device using a membrane cutoff 300k, (catalog No. OC300C33, Pall Gelman Laboratory) which by centrifugal force (up to x7500g, for 45 min) drive the free cytokine through the membrane (the removed, free cytokine is referred to as the "Filtrate"). HBsAg entrapped cytokine remained on the filter.

The addition of PBS and vortex resulted in recovery of the entrapped HBsAg particle (referred to as the "Retentate").

A second ultrafiltration was employed in order to remove remaining free cytokine from the retantate which was followed by the addition of 1ml PBS onto the membrane and vortexing to obtain a pure preparation containing HBsAg entrapped cytokine (referred to as the "Wash Retentate").

A further was resulted in a filtrate (referred to as the "Wash Filtrate").

Entrapment determination:

The *Retentate*, the *Filtrate*, *Wash Retentate* and *Wash Filtrate* were analyzed for cytokine entrapment by three methods:

1. By the Lowry method (Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. J. Biol. Chem. (1951), **193**:265-275). Total protein content (HBsAg, HBsAg + cytokine, or free cytokine) was determined after solubilization of the HBsAg particles with SDS (5% final concentration).

2. By absorbance at 280 nm after complete solubilization with SDS (final concentration 5%).

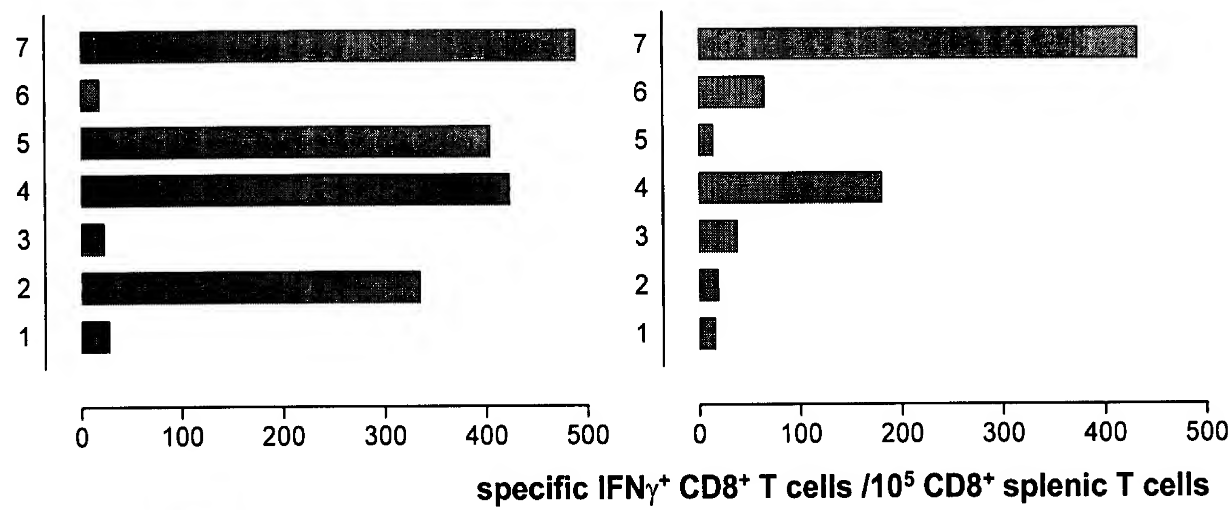
3. By denaturing polyacrylamide gel electrophoresis ("PAGE", in the presence of SDS and β-mercaptoethanol). Calibration curve for quantification by PAGE was obtained by the use of HBsAg particles and cytokines (IL-2 or IFN-α) standardized according to the Lowry method. Peptide composition was determined by SDS-PAGE, followed by "Simply Blue Safe Stain" (Invitrogen) using protein markers for molecular weight (MW) determination.

4. Bioassay- (a) IL-2 encapsulation efficiency was determined by measuring thymidine incorporation in CTLL-2 murine cell in the presence of IL-2 [Kedar E., *et al.* Delivery of cytokines by liposomes- Preparation and characterization of interleukin-2 encapsulated in long-circulating sterically stabilized liposomes. *J. Immunother.* **16**:47–59 (1994); Bishara A., *et al.* A short human and mouse MLR assay utilizing lymphokine (IL-2, IL-3) secretion as an early activation event. *Transplantation*; **51**: 1104-1109 (1991)].

(b) INF-α encapsulation was determined by the Zwei Test [Meager A., Establishment of new and replacement World Health International Standards for human INF-alpha and omega. J. of Immunological Methods **257**:17-33 (2001)].

Table 1 summarized the entrapment and bioactivity (% from theoretical bioactivity) of IL-2, while Table 2 summarized the entrapment and bioactivity (% from theoretical bioactivity), of INF-α, with the different entrapment methods.

MHC class I-restricted CTL response in BALB/c mice



group	HBsAg (2 μg/mouse)	pep (50 μg/mouse)	IL-2
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	+	-	+
6	-	+	+
7	+	+	+

K^d/HBcAg-derived peptide (pep):

LVVSYVNTNMGLKFRQLLWF

Exhibit D.

CURRICULUM VITAE

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EDUCATION

1966 - 73	Premedical and Medical School
1973	Graduation
1973 - 74	Internships
1974	License to practice

PROFESSIONAL EXPERIENCE

1974 - 75	Residency/Clinical Pediatrics, University of British Columbia, Vancouver, Canada
1975	Medical Thesis, Free University of West-Berlin, Germany
1976 - 77	Fellowship, Imperial Cancer Research Fund, London, UK
1977 - 78	Fellowship, Radiobiological Institute, Rijswijk, NL
1978 - 81	Research Associate, Immunological Research Unit, Free University of West-Berlin, Germany
1982 - 83	Fellowship, Ontario Cancer Institute, Toronto, Ontario, Canada
1984 - 92	Associate Professor, Institute for Medical Microbiology, University of Ulm, Germany
1985	Lectureship in Microbiology and Immunology
1987 - 88	Sabbatical, Ontario Cancer Institute, Toronto, Canada
1990	Specialty License in Medical Microbiology
1991-2002	Professor at the Institute for Medical Microbiology, University of Ulm, Germany

Prof. Dr.med. Jörg Reimann (geb. Kulenkampff)

List of Publications

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3. **Reimann, J.** and Burger, H. *In vitro* proliferation of haemopoietic cells in the presence of adherent cell layers. I. Culture conditions and strain dependance. *Exp.Hemat.* **7**:45 (1979).

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5. Diamantstein, T., Willinger, E. and **Reimann, J.** T-supressor cells sensitive to cyclophosphamide and to its *in vitro* active derivative 4-hydroperoxycyclophosphamide control the mitogenic response of murine splenic B cells to dextran sulphate. *J.exp.Med.* **150**:1571 (1979).

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7. **Reimann, J.** and Diamantstein, T. "Self-reactive" T cells. I. *In vivo* reaction of T cells to transferred polyclonally activated syngeneic and autologous lymphoblasts. *Immunobiol.* **157**:437 (1980).

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10. **Reimann, J.** and Diamantstein, T. "Self-reactive" T cells. IV. "Self-reactive" T cells induce polyclonal differentiation of IgM-producing B cells *in vitro* and *in vivo*. *Immunobiol.* **159**:215 (1981).

11. **Reimann, J.** and Diamantstein, T. "Self-reactive" T cells. V. T cell-mediated suppression of B cell responsivness to LPS. *Immunobiol.* **159**:228 (1981).

12. **Reimann, J.**, Kaufmann, S.H.E. and Diamantstein, T. Lymphoblastogenesis stimulates proliferation of interacting Lyt-1 T cells in a syngeneic system. *Eur.J.Immunol.* **11**:604 (1981).

13. **Reimann, J.** and Diamantstein, T. Interleukin-2 allows *in vivo* induction of anti-erythrocyte autoantibody production in nude mice associated with the injection of rat erythrocytes. *Clin.Exp.Immunol.* **43**:641 (1981).

14. **Reimann, J.** and Diamantstein, T. Studies on T-lymphocytes activation. I. Is competence induction in thymocytes by phorbol myristate acetate an accessory cell-independent event? *Immunology* **43**:183 (1981).

15. **Reimann, J.** and Miller, R.G. Differentiation from precursors in athymic nude mouse bone marrow of unusual spontaneously cytolytic cells showing anti-self-H-2 specificity and bearing T cell markers. *J.exp.Med.* **158**:1672 (1983).

16. **Reimann, J.** and Miller R.G. Generation of autoreactive cytotoxic T lymphocytes under limiting dilution conditions. *J.Immunol.* **131**:2128 (1983).

17. **Reimann, J.** and Miller R.G. Polymorphism and MHC gene function. *Dev.Comp.Immunol.* **7**:403 (1983).

18. **Reimann, J.**, Ehmann, D. and Miller R.G. Differential binding of lectins to lymphopoietic and myelopoietic cells in murine marrow as revealed by flow cytometry. *Cytometry* **5**:194 (1984).

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23. Heeg, K., **Reimann, J.**, Heit, H., Heit, W. and Wagner, H. Host-reactive cytotoxic T lymphocyte precursors in long-lived fully allogeneic mouse bone marrow chimeras. *Scand.J.Immunol.* **23**:201 (1986).

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28. **Reimann, J.** and Bellan A.. Use of Vß8 genes in splenic Lyt-2^+ cytotoxic lymphocyte precursors reactive to bm1 or bm14 alloantigen in individual B6 mice. *Eur.J.Immunol.* **16**:1597 (1986).

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31. **Reimann, J.**, A. Bellan and Kabelitz, D. Antigen-presenting T cells. I. Class I alloantigen (bm1)-bearing T lymphoblasts efficiently stimulate a primary clonal response of Lyt-2^+ cytotoxic precursors of B6 origin. *J.Immunol.* **138**:1042 (1987).

32. Kabelitz, D., K.-H. Enssle, Fleischer, B., and **Reimann, J.** Antigen presenting T cells. II. Clonal response of alloreactive and virus-specific self-restricted human cytotoxic T cells stimulated by T lymphoblasts. *J.Immunol.* **138**:45 (1987).

33. Brucker, C., **Reimann, J.**, Wagner, H. and Kabelitz, D. Clonal activation of cytotoxic lymphocyte precursors by monoclonal anti-CD3 antibody: analysis of feeder cell requirements. *Immunology Letters* **14**:121 (1987).

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